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ORIGINAL ARTICLE

Citrus limon L. Gel Stimulate Lymphocytes and Interleukin-10 Expression in Traumatic Diabetic Oral Ulcers

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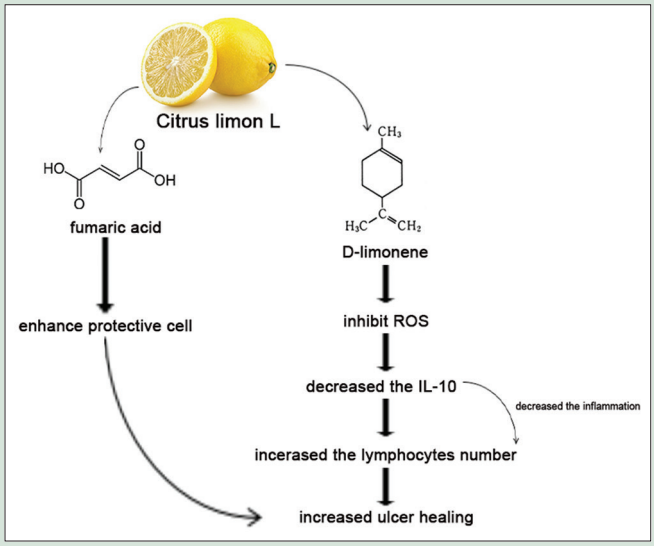
ABSTRACT

Background: *Citrus limon* L. and its peel report anti-inflammatory, antioxidant, anti-fungal, and anti-bacterial properties. However, the effects of *C. limon* L. gel to treat traumatic oral ulcers on diabetic conditions have yet to be investigated. **Purpose:** This study aims to discover the potent effect of *C. limon* L. on traumatic diabetic oral ulcers, in terms of both lymphocytes and interleukin (IL)-10 expression. **Materials and Methods:** *C. limon* L. gel was made from *C. limon* L. peel and was extracted by means of steam distillation. Twenty male, diabetic Wistar rats with traumatic ulcers on the inferior labial mucosa were divided into four groups (n = 5). The control groups were treated with 3% carboxylmethyl celullosa natrium and the treatment groups were treated with *C. limon* L. gel. Each treatment was administered once a day for either 5 or 7 days. Lymphocytes were analyzed by means of hematoxylin and eosin staining and IL-10 expression by immunohistochemistry staining. An independent t-test ($P < 0.05$) was used to analyze and determine the number of lymphocytes and IL-10 expression. **Results:** The number of lymphocytes and IL-10 expression in the treatment groups on the fifth and 7th days was higher than those in the control groups ($P < 0.05$). **Conclusion:** The essential oil in *C. limon* L. peel can be used to treat traumatic diabetic oral ulcers, as it is able to stimulate the number of lymphocytes and IL-10 expression.

Key words: *Citrus limon* L, diabetes mellitus, interleukin-10, lymphocytes, traumatic ulcer

SUMMARY

- Fumaric acid and D-limonene are the dominant components of the essential oil in *C. limon L.* D-limonene, as an antioxidant, acts by donating hydrogen ions (H+) and increasing the level of antioxidant enzymatic endogen, which suppress reactive oxygen species (ROS) levels. Decreased ROS can suppress pro-inflammatory cytokine, such as interleukin-10 by inhibiting lymphocyte activation and proliferation. Fumaric acid can increase the level of endogen antioxidant glutathione (GSH), which enhances cells' protection against oxidative stress. Both mechanisms stimulate and increase ulcer healing.



Abbreviations Used: 3% CMC-Na: 3% carboxylmethyl celullosa natrium, CAT: Catalase, CCL5: Chemokine (C-C motif) ligand 5, CCL20: Chemokine (C-C motif) ligand 20, FGF: Fibroblast growth factor, GC-MS: Gas chromatography–mass spectrometry, GPx: Glutathione peroxidase, GSH: Antioxidant glutathione, HO-1: Heme oxygenase-1, IL-6: Interleukin 6, IL-10: Interleukin-10, MDA: Malondialdehyde, NF-kB: Nuclear factor kappa beta, NSAID: Nonsteroid anti-inflammation drug, ROS: Reactive oxygen species, SOD: Superoxide dismutase, Th1: T-helper 1, Th17: T-helper 17, TNF-α: Tumor necrosis factor α.

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INTRODUCTION

Ulcers are one of the most common oral lesions. They are characterized by a local defect, with epithelial surface discontinuity that extends to the basalis membrane. Traumatic ulcers, which have a high prevalence in the oral cavity, are usually caused by mechanical injuries, heat, or direct chemical contact with the mucosa. This condition is increasingly found in people with diabetes.[1,2] When irritant factors are eliminated, oral traumatic ulcers commonly heal in 1–2 weeks due to the keratinization and re-epithelization processes

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within the oral mucosa. However, compared to normal people, individuals with diabetes mellitus (DM) will experience delays in, or even the failure of, the healing process. This condition is passed by several complex, intricate processes, such as microangiopathy, neuropathy, and cell-immunity dysfunction.[2]

At present, topical corticosteroid or topical nonsteroid anti-inflammatory drugs are used to treat oral traumatic ulcers and to accelerate healing. However, these drugs can cause side effects, which represent a contraindication for people in whom DM causes other problems. Therefore, natural medicine can provide an alternative beneficial curative treatment to overcoming this problematic situation.[3]

The use of herbal medicines has been reported among Indonesians, in response to drugs becoming more expensive and the rate of chemical-drug resistance increasing. In dentistry, for example, the essential oil of *Citrus limon* L. peel is under development as an alternative therapeutic treatment for oral traumatic ulcers. *C. limon* L. is one of the most common citrus species, whose peel is composed of two layers: the flavedo and albedo. The essential oil of the peel resides in the flavedo, which is widely used for its flavor and aroma in industrial processes. The albedo, a spongy and cellulose layer located under the flavedo, is the main component of the peel.[3-6]

This study evaluates the role of East Java's *C. limon* L. gel as an alternative therapeutic treatment for the accelerated healing of traumatic diabetic oral ulcers in animal models. Analysis involved observation of the number of lymphocytes and interleukin (IL)-10 expressions after treatment once a day for either 5 or 7 days, which represents the end of the inflammation stage and the beginning of proliferation.

MATERIALS AND METHODS

Experimental design

The animal models were 20 adult, male, diabetic Wistar rats (2–3-month old), weighing 200–250 g. A diabetic condition was induced by injecting streptozocin (Sigma Aldrich, S0130, Saint Louis, Missouri, USA) (IP) (50 mg/kg dose), which was then confirmed by blood glucose levels of 200 mg/dl in the 3 days after administration. During sampling, the rats were randomly split into four groups ($n = 5$): Control groups were treated with 3% carboxylmethyl celullosa natrium (CMC-Na) for 5 days (Group A) and 7 days (Group C), while the treatment groups were treated with *C. limon* L. *gel* for 5 days (Group B) and 7 days (Group D).

All preparation procedures adhered to the Ethical Committee of the Faculty of Dental Medicine, Universitas Airlangga, with registered number 109/HRECC. FODM/VII/2017.

Identification and preparation of Citrus limon L. *gel*

C. limon L. was collected from the Subtropical Fruit Research Institute, East Java, Indonesia. The essential oil was produced in the same manner as that used in the previous studies by Ganesha *et al*. and Surboyo *et al*.[3,5] The essential oil of the peel was extracted by steam distillation and turned into gel with 3% CMC-Na. It was also subjected to gas chromatography–mass spectrometry (GC-MS) examination to identify the compounds that comprise the essential oil. GC-MS analysis was equal to that in the previous study conducted by Surboyo *et al*.[3]

Confirmation of traumatic ulcer

A traumatic ulcer was produced through 1s of pressure-less contact, using a 5 mm burnisher that had been previously heated for 30 s,[3] after which the diabetic condition was confirmed.[7] Clinically, an oral ulcer appears as a yellowish-white base with a reddish edge, 24 h later. At this point, the treatment was topically administered to all groups.

Histological examination

The rats involved were sacrificed using a lethal dose of inhaled chloroform. The inferior labial mucosae of these rats were then collected and analyzed using hematoxylin and eosin staining (Santa Cruz Biotechnology, Santa Cruz, California, USA) to determine the number of lymphocytes under a Nikon H600 L (Nikon, Tokyo, Japan) and a light microscope that was equipped with a DS Fi2 300 megapixel camera at a magnification of ×400. Immunohistochemistry staining was applied to determine IL-10 expression using the monoclonal antibody IL-10 (Santa Cruz Biotechnology Inc., Dallas, Texas, USA), which was examined under a light microscope that was equipped with a DS Fi2 300 megapixel camera, with the magnification set at ×1000.

Statistical analysis

The resulting data were analyzed by independent *t*-test ($P < 0.05$) using SPSS software version 21 (IBM, New York, USA).

RESULTS

The taxonomic test identified the lemon fruit used in this research as *C. limon* L. In addition, Burm. f and GC-MS analysis revealed that the essential oil was composed of 51 compounds, which aligns with the findings of the previous studies.[3]

The hematoxylin and eosin staining [Figure 1] revealed lymphocytes in the form of large cells that were dark purple, each with a nucleus occupying almost all the cell and a thin cytoplasm that was reddish in color. The immunohistochemistry staining [Figure 2] demonstrated that lymphocytes and macrophages that express IL-10 reacted with diaminobenzidine and appeared brown at ×1000 magnification, in five different fields. This was examined by two experts.

The mean number and standard deviation (SD) (mean ± SD) of the lymphocytes and IL-10 expression are presented in Table 1. The lymphocytes and IL-10 expression of the treatment groups (Group B and Group D) were significantly higher than those of the control groups (Group A and Group C). The number of lymphocytes in Group B (24.00 ± 1.23) was higher than in Group A (9.60 ± 1.67) ($P = 0.000$), while IL-10

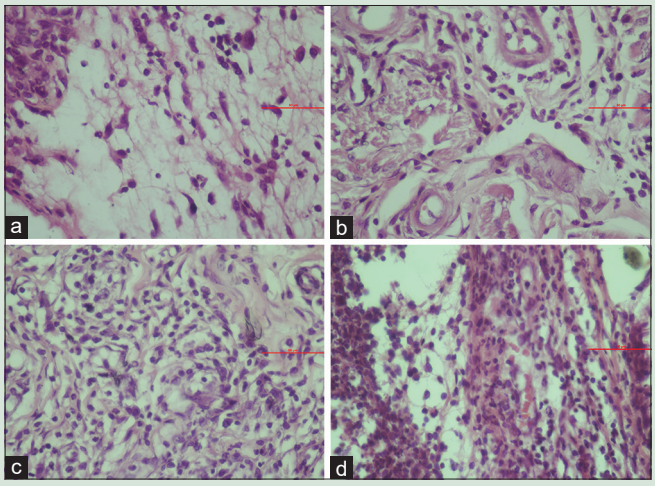

Figure 1: Histological illustration of lymphocytes on the inferior labial mucosa (a) Group A, (b) Group C, (c) Group B, and (d) Group D

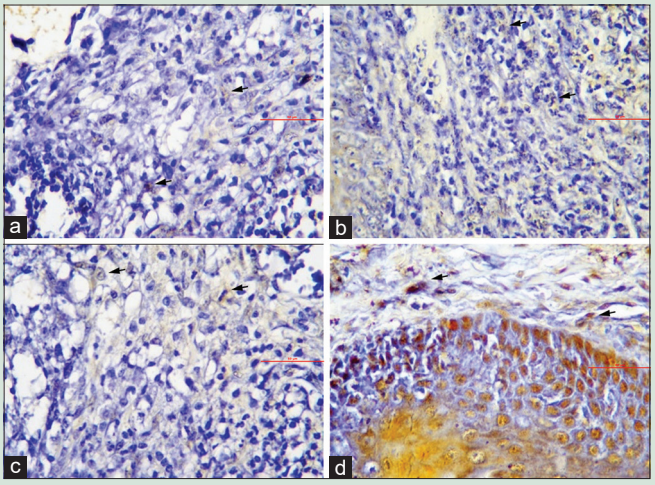

Figure 2: Interleukin-10 expression with immunohistochemistry staining on the inferior labial mucosa (a) Group A, (b) Group C, (c) Group B, and (d) Group D

expression in the Group B (14.60 ± 3.2) was higher than in the Group A (5.60 ± 3.05) ($P = 0.002$).

The number of lymphocytes in the Group D (27.80 ± 1.79) was higher than in the Group C (11.0 ± 2.55) ($P = 0.000$), and IL-10 expression in the Group D (16.60 ± 2.07) was higher than in the Group A (510.40 ± 3.28) ($P = 0.010$).

DISCUSSION

The traumatic ulcer healing process involves several successive phases which include hemostasis, inflammation, proliferation, and remodeling. The presence of lymphocytes in the chronic inflammation stage plays an important role in destroying antigens, as well as in the formation of immunoglobulin, which acts as an antibody. Lymphocytes secrete lymphokine, which is capable of stimulating macrophage aggregations. Along with macrophages, a fibroblast growth factor (FGF) and IL-10 were also secreted by the lymphocytes. FGF stimulates the formation of granulation tissue and is also involved in re-epithelization and fibroblast proliferation. IL-10, meanwhile, provides a negative feedback about pro-inflammatory cytokine, which inhibits macrophage and neutrophil activation. Therefore, the prolonged inflammation stage does not continuously occur.[2,3,8-10]

The results of this research reveal that Group A and Group C demonstrated the lowest incidence of lymphocyte and IL-10 expression. This is due to the use of 3% CMC-Na as a placebo, which was given to the control group. CMC-Na lacks any active compound and acts as a covering agent. In the traumatic ulcer healing process, the covering agent inhibits secondary infection. With no therapeutic effect, the amount of lymphocytes was the lowest of all the groups.[2,9]

The mean lymphocyte number and IL-10 expression was higher in the treatment groups than in the control group for those treated both once a day for 5 and 7 days. This was possibly due the topical application of *C. limon* L., which can decrease the amount of reactive oxygen species (ROS). ROS is a reactive compound that can destroy any type of biomolecule, such as proteins, lipids, carbohydrates, and nucleic acids. It also induces pro-inflammatory cytokine, which leads to prolonged inflammation and, in turn, can cause protracted wound healing. ROS levels are known to be higher in people suffering from DM. The human body usually already contains endogen antioxidants, such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase. However, under conditions within which ROS continually increases, the human body is unable to neutralize the effect of ROS.[6,9,11]

Table 1: Mean and standard deviation of lymphocytes and interleukin-10 expression in each group

Group	Mean±SD	
	Lymphocytes	IL-10
Group A	9.60±1.67[a]	5.60±3.05[b]
Group B	24.00±1.23[a]	14.60±3.2[b]
Group C	11.0±2.55[c]	10.40±3.28[d]
Group D	27.80±1.79[c]	16.60±2.07[d]

[a,b,c,d]Different superscript denotes significant difference $P<0.05$ using independent *t*-test. SD: Standard deviation; IL: Interleukin

The essential oil used in this study was discovered to have 51 composed substances. The most dominant substance was fumaric acid, which produces anti-inflammatory and antioxidant effects. As fumaric acid can increase the level of endogen GSH, it strengthens the induction process of anti-inflammatory protein heme oxygenase-1, which, in turn, enhances cells' protection against oxidative stress. D-limonene, which is a special compound found only in *C. limon* L., has antioxidant, anti-inflammatory, and antibacterial properties. As an antioxidant, D-limonene acts by donating hydrogen ions (H+) and increasing the level of antioxidant enzymatic endogen, which suppress ROS levels. Linalool-L, whose concentration was 8.51%, acts as an antioxidant modulating malondialdehyde and lipid peroxidation indicator of ROS, which increase the level of the endogen antioxidant GSH. Thus, the presence of fumaric acid, D-limonene, and linalool-L in the essential oil could help maintain the balance of ROS inside the human body.[10,12-14]

The increase of lymphocytes was possibly due to D-limonene's anti-inflammatory properties. These properties act by decreasing the amount of pro-inflammatory cytokine, such as the tumor necrosis factor α and IL-6, as they can inhibit the activation and proliferation of lymphocytes. The increased expression of IL-10 is thought to occur through several receptor mechanisms involving limonene and the immune cells, which then lead to endocytosis and the nuclear targeting of limonene in the immune cells. One such proposed molecular mechanism is via the nuclear factor kappa beta (NF-κB), as limonene is thought to suppress the inflammatory response by inhibiting the expression or activation of NF-κB.[15] NF-κB is an important modulator of chemokine transcription, such as chemokine (C-C motif) ligand 5 (CCL5) and chemokine (C-C motif) ligand 20 (CCL20), which play important roles in the migration of T-helper (Th) 1 and Th17 cells. Therefore, inhibiting the activation of NF-κB induces a decrease in Th17 cell infiltration in the area of the ulcer.[3,5,10,12,13,16]

In addition, the antimicrobe effect on *C. limon* L. gel can also inhibit secondary infection in chronic traumatic ulcers. The antibacterial mechanism functions (works) by accumulating toxins in bacterial cell membranes, changing the composition of the fatty acid in bacterial cell membranes, and causing leakage through proton motive force. The antifungal Z-citral mechanism can inhibit the development of *Candida albicans*, *Candida glabrata*, and *Candida tropicalis*. Terpenoid compounds, which can inhibit ergosterol production, were also present. This will therefore disturb the permeability of the fungus and inhibit growth.[4,16,17] Z-citral also functions as an antidiabetic by controlling progression of the impaired glucose tolerance in individuals with DM. In so doing, this compound regulates insulin resistance, through the inhibition of alpha amylase.[4,6,12,13]

CONCLUSION

Topical application of East Java's *C. limon L.* gel can increase lymphocytes and the expression of IL-10 in the traumatic ulcers of diabetic Wistar rats. This is because ROS helps to produce a protection on lymphocytes in considerable numbers, under diabetic conditions.

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Conflicts of interest

There are no conflicts of interest.

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